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INFLUENCE OF pH ON THE REMOVAL OF PYRIDOXAL 5′-PHOSPHATE FROM PHOSPHORYLASE *b*

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A method to break the pyridoxal 5′-phosphate (PLP)-phosphorylase *b* bond using hydroxylamine and slightly acid pH is put forward and described in the present paper. This method does not involve drastic conditions or deforming reagents. The influence of pH and protein concentration on the removal of PLP from phosphorylase has also been studied, resulting in an order of −0.3 with respect to the enzyme, a value that implies a complex reaction. An additional conclusion is that an increase in the protein concentration entails better protection of the enzyme from attack by hydroxylamine.

1. Introduction

The structure of phosphorylase *b* is strongly affected by pH, remaining stable only in the pH range 5.0–9.5 [1]. The study performed by Kasvinsky and Meyer [2] on alterations in the maximum enzymatic activity with pH shows that this activity is related to ionization of two groups with pK 6.1 and 7.3. The first pK has been assigned to the second proton of the phosphate group present in the coenzyme and the second to ionization of the imidazoyl ring of an enzyme histidyl residue.

Cortijo et al. [3] have observed that fluorescence of the protein moiety has a constant intensity in the pH range 5.0–9.5, while there are changes in the fluorescence of the coenzyme around pH 6.

Pyridoxal 5′-phosphate (PLP) is present in all α-glucan phosphorylases, being essential to their activity [4]. In phosphorylase *b* from rabbit skeletal muscle, PLP is bound to Lys-679 [5] with a stoichiometry of one PLP molecule per enzymatic subunit, through a Schiff base derivative within a hydrophobic environment totally inaccessible to the solvent with the possible exception of the 5′-phosphate group [6–8].

PLP stabilizes the quaternary structure of phosphorylase *b* which is a prerequisite for activation [9]. If we consider all the groups present in PLP, only 5′-phosphate and the nitrogen atom of the pyridine ring can be involved in the catalytic process [10–12]. However, the nitrogen atom probably acts merely as an anchor point between PLP and the enzyme [11].

The possibility of an exchange between the phosphate group of the cofactor and the substrates, P_i and glucose 1-phosphate, was ruled out by Illingworth et al. [13] using labelled substrates.

Feldmann and Hull [14], using ^{31}P-NMR spectroscopy, state that the 5′-phosphate group of PLP is present in the monoanionic form in native phosphorylase and in the dianionic form when the activator and substrates are both present. On this basis, Helmreich and Klein [15] propose that the phosphate group of PLP functions as an acceptor and donor of protons in the catalytic process.

In the present paper, the removal of PLP from

phosphorylase *b* has been carried out using hydroxylamine. The process has been followed using light-scattering as well as spectroscopic techniques (ultraviolet absorption and fluorescence). The results show that elimination of PLP from phosphorylase *b* is a complex process strongly affected by pH and enzymatic concentration.

2. Materials and methods

Phosphorylase *b* was obtained from rabbit skeletal muscle by the method of Krebs et al. [16]. The enzyme was recrystallized three times before use. The enzyme concentration was measured spectrophotometrically using an extinction coefficient, $E^{1\%}_{cm}$, of 13.2 at 280 nm [17], and its activity was determined in the direction of glycogen breakdown following the procedure first described by Helmreich and Cori [18].

The buffer solution used in all experiments was 50 mM glycylglycine, 50 mM KCl and 0.2 mM EDTA adjusted to the desired pH. The molecular weight of the monomer was taken as 97 500 [5].

The reaction between hydroxylamine and the enzyme was started by mixing identical volumes of both reagents dissolved in the buffer previously described at a fixed pH.

To obtain its Schiff base derivative, PLP (approx. 10^{-3} M) was reacted with excess *n*-hexylamine (0.1 M in ethanol) to prevent decomposition. This derivative was diluted 10-fold into ethanol/water mixtures so that the final solution had the indicated percentage of water; pH was adjusted with HCl. pH was determined using a pH-meter calibrated with aqueous buffer solutions of pH 4.01 ± 0.01 and 7.00 ± 0.01 at 25 °C. Therefore, values obtained for nonaqueous solvents have been corrected according to data provided by Bates et al. [19] for this kind of measurement in ethanol/water mixtures.

Light-scattering measurements were performed using an FICA model 42000 light-scattering photometer equipped with a thermostatted xylene bath. These measurements were made at an angle of 90°, since the correction factor due to dissymmetry is negligible [20,21].

Calculation of molecular weights was accomplished using the equation:

$$\frac{kc}{\Delta R_{90°}} = \frac{1}{\overline{M}_{w,app}}$$

where $\Delta R_{90°}$ is the Rayleigh ratio of the solution at an angle of 90°, c the protein concentration in mg/ml, $\overline{M}_{w,app}$ the weight average molecular weight and k is given by:

$$k = 2\pi^2 n^2 (dn/dc)^2 / 1000 N_A \lambda^4$$

where n is the refractive index of the solvents, dn/dc the specific refractive index increment of the protein and λ the wavelength of primary light in vacuum (546 nm).

Under our conditions, the apparent weight average molecular weight is identical to the weight average molecular weight, since the second virial coefficient is practically zero for phosphorylase *b* [21].

Fluorescence and light-scattering experiments were performed at the same temperature. Fluorescence studies were carried out in an FICA model 55 spectrofluorimeter, recording the change in fluorescence of the characteristic PLP band: excitation at 425 nm and emission at 535 nm [3]. The slit width was 7.5 nm

Absorption experiments were performed using a Zeiss DMR 11 double-beam spectrophotometer at 25.0 ± 0.1 °C. Quartz cuvettes of 1 cm path length were used. The slit width was less than 0.6 nm.

3. Results

At 28 °C and in the absence of hydroxylamine, phosphorylase *b* remains in the dimeric form at any enzyme concentration [22]. The presence of 0.1 M hydroxylamine causes alterations in the molecular weight, an effect enhanced by a decrease in the pH of the solution (fig. 1). This variation points clearly to the existence of two processes: firstly, monomeric species are formed (due to removal of PLP from the protein) resulting in a drop of the molecular weight; secondly, apophosphorylase undergoes self-aggregation, since the quaternary structure of apophosphory-

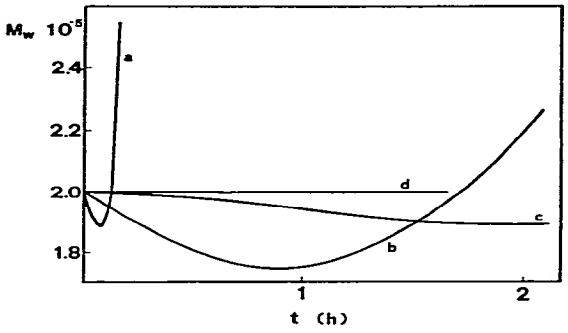

Fig. 1. Molecular weight of the enzyme (1 mg/ml) plotted as a function of incubation time in 0.1 M hydroxylamine at 28.5 °C and different pH: (a) 5.4, (b) 6.0, (c) 6.3, (d) 7.0 and 7.5

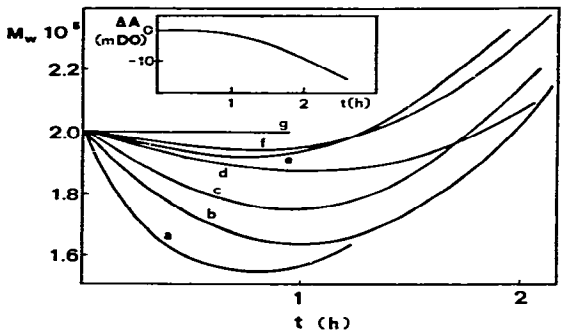

Fig. 2. Molecular weight of the enzyme as a function of incubation time in 0.1 M hydroxylamine at 28.5 °C and pH 6.0. Enzyme concentration (mg/ml): (a) 0.4, (b) 0.6, (c) 1.0, (d) 1.5, (e) 2.0, (f) 2.1, (g) 3.0. Inset: absorption band of the enzyme (0.6 mg/ml) centred at 256 nm plotted vs. incubation time in 0.1 M hydroxylamine. Temperature 28.5 °C.

lase *b* is unstable at high temperatures [9]. Parallel activity assays indicated a decrease in its values with incubation time in 0.1 M hydroxylamine.

The lower the pH, the greater its influence becomes upon the change in molecular weight, which means that acidity of the solution favours both the attack of NH_2OH on PLP and the self-aggregation process of apophosphorylase.

Since the effect of enzyme concentration upon the process of removal of the cofactor has not been described so far, experiments have been performed at a fixed pH and several protein concentrations (fig. 2). pH 6.0 has been chosen, because under these conditions we obtain moderate as well as significant variations of the molecular weight. Moreover, no enzymatic denaturation due solely to pH is observed at this pH value and the activity is higher than 80% [3].

The change in molecular weight has been plotted in fig. 2 vs. incubation time in 0.1 M hydroxylamine at the pH previously mentioned and different enzyme concentrations. It must be emphasized that an increase of the enzyme concentration entails better protection from the attack by hydroxylamine.

The rate of monomerization of the enzyme produced by 0.1 M NH_2OH at pH 6.0 can be expressed as:

$$v = -\frac{d[D]}{dt} = k[\text{Enzl}]^a[NH_2OH]^b \qquad (1)$$

where [D] is the concentration of dimeric species that, at $t = 0$, can be taken as the total protein concentration, since under these conditions, the native enzyme is to be found in the dimeric form [21,22]. The value of [D] is given by the expression:

$$[D] = \frac{c}{2M_m}\left(\frac{\overline{M}_w}{M_m} - 1\right) \qquad (2)$$

where c is the enzyme concentration in mg/ml, M_m the molecular weight of the monomer and $\overline{M}_w$ the weight average molecular weight.

The expression that gives the initial rate can be written as:

$$v_0 = -\frac{c}{2M_m^2}(d\overline{M}_w/dt)_0 = k\left(\frac{c}{2M_m}\right)^a [NH_2OH]_0^b \qquad (3)$$

From eq. 3, we obtain:

$$-(d\overline{M}_w/dt)_0 = \left[2kM_m^2[NH_2OH]_0^b/(2M_m)^a\right]c^{a-1} \qquad (4)$$

If we convert eq. 4 to the logarithmic form:

$$A' = -\log(-d\overline{M}_w/dt) = \text{constant} + (1-a)\log c \qquad (5)$$

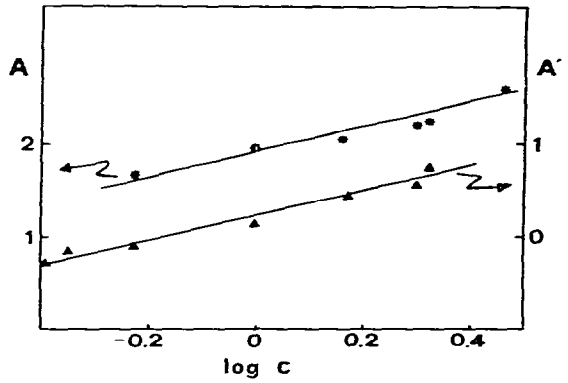

Fig. 3. Determination of the order of reaction for the dissociation of the PLP-enzyme bond with respect to the enzyme concentration in 0.1 M hydroxylamine, at 28.5 °C and pH 6.0. (▲) Calculated from light-scattering measurements. (*) calculated from fluorescence measurements (see text).

we obtain an equation that allows calculation of the order of reaction with respect to the enzyme from the initial slopes of the curves in fig. 2, namely $(d\bar{M}_w/dt)_0$. These values have been plotted in fig. 3, giving a slope of 1.3 which means that the order with respect to the enzyme is -0.3.

The results mentioned above indicate a decrease of the molecular weight, but do not prove conclusively that removal of PLP has been accomplished. For this reason, we have undertaken a spectroscopic study of the cofactor under different conditions, using ultraviolet absorption and fluorescence techniques.

The protein has an absorption band at 280 nm and two others at 330 and 415 nm in an approximate ratio of 100 : 5.3 : 0.4 [3]. These two smaller bands are assigned to PLP [7]. Excitation at 330 and 415 nm gives rise to an emission fluorescence at 525 nm, a peculiarity that has been explained as indicating that PLP is bound through a Schiff base to a lysine residue within a hydrophobic environment.

The decrease of the enzyme molecular weight in the presence of 0.1 M hydroxylamine occurs parallel to a drop in the absorption maximum at 256 nm, assigned to the cofactor (see inset in fig. 2). This fact is ascribed to removal of PLP, in line with the results of Shimomura and Fukui [23]. Therefore, the spectral properties of PLP in phosphorylase *b* disappear when the enzyme is incubated with hydroxylamine.

Fluorescence intensity also diminishes with incubation time (figs. 4 and 5) but, in contrast to the change in molecular weight, the curves that show the fall in fluorescence always have a negative slope, a logical result since this technique only detects cleavage of the PLP-enzyme bond.

The influence of pH upon the process of removal of PLP was studied using fluorescence (fig. 4), as performed previously with light scattering.

When the resolution of phosphorylase *b* from PLP is thoroughly studied at pH 6.0 as a function of enzyme concentration (fig. 5), it can be observed that PLP reactivity is strongly influenced by protein concentration, as occurred using light scattering. The drop in fluorescence intensity with incubation time takes place because of the decrease in the amount of PLP bound to the enzyme, the rate of reaction of PLP with hydroxylamine being given by:

$$v = -(d[\text{PLP-Enz}]/dt) = k'[\text{PLP-Enz}]^a[\text{NH}_2\text{OH}]^b \quad (6)$$

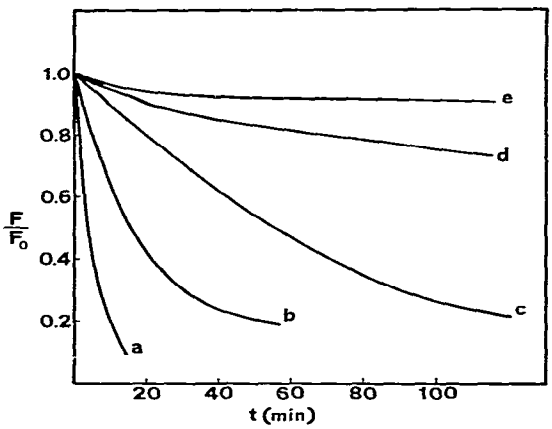

Fig. 4. Fluorescence ratio of the enzyme (1 mg/ml) plotted vs. incubation time in 0.1 M hydroxylamine, at 28.5 °C. pH: (a) 5.4, (b) 5.8, (c) 6.0, (d) 6.3, (e) 7, 7.5 and 8.3.

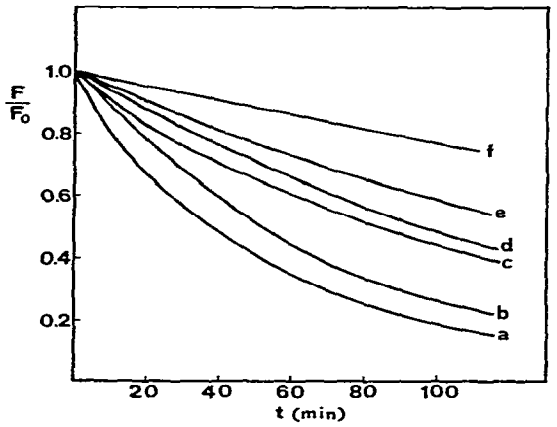

Fig. 5. Fluorescence ratio of the enzyme as a function of incubation time in 0.1 M hydroxylamine, at 28.5 °C and pH 6.0. Enzyme concentrations (mg/ml): (a) 0.6, (b) 1.0, (c) 2.0 (d) 2.1, (e) 3.0.

where [PLP-Enz] is the concentration of PLP bound to the enzyme, which can be initially expressed as:

$$[\text{PLP-Enz}]_0 = c/M_m \tag{7}$$

At low protein concentrations and whenever the absorption at the excitation wavelength is negligible (there is no internal quenching), fluorescence can be considered proportional to the amount of PLP bound to the enzyme. For this reason, the excitation wavelength was 425 nm instead of 330 nm, because the latter would only allow one to work at an enzyme concentration of 0.1 mg/ml.

In this case, and independently of experimental conditions, we can define the following fluorescence ratio:

$$\frac{F}{F_0} = \frac{k'[\text{PLP-Enz}]}{k'[\text{PLP-Enz}]_0} = \frac{[\text{PLP-Enz}]}{c/M_m} \tag{8}$$

where F and F_0 are the fluorescence intensities for incubation times t and zero, respectively.

The initial rate will be given by the expression:

$$-\left(\frac{\mathrm{d}F/F_0}{\mathrm{d}t}\right)_0 = \frac{k'}{M_m^{a-1}} c^{a-1}[\text{NH}_2\text{OH}]_0^b. \tag{9}$$

If we convert eq. 9 to the logarithmic form:

$$-\log\left[-\left(\frac{\mathrm{d}F/F_0}{\mathrm{d}t}\right)_0\right] = \text{constant} + (1-a)\log c \tag{10}$$

an equation that allows one to calculate the order of the reaction with respect to the enzyme if the values of $(\mathrm{d}(F/F_0)/\mathrm{d}t)_0$ are previously determined by measuring the initial slopes of the curves shown in fig. 5.

We obtain an order of -0.3 for this reaction (fig. 3), the same as that we obtained using light scattering, which proves that monomerization of the enzyme cannot be separated from the removal of PLP.

The influence of pH can be analyzed if we take into account that:

$$v = -\frac{\mathrm{d}[\text{PLP-Enz}]}{\mathrm{d}t}$$

$$= k''[\text{PLP-Enz}]^a[\text{NH}_2\text{OH}]^b[\text{H}^+]^n \tag{11}$$

When $t = 0$, we obtain:

$$-\log\left[-\left(\frac{\mathrm{d}F/F_0}{\mathrm{d}t}\right)_0\right] = \text{constant} + n\text{pH} \tag{12}$$

Plotting the first term of eq. 12 vs. pH (fig. 6), it

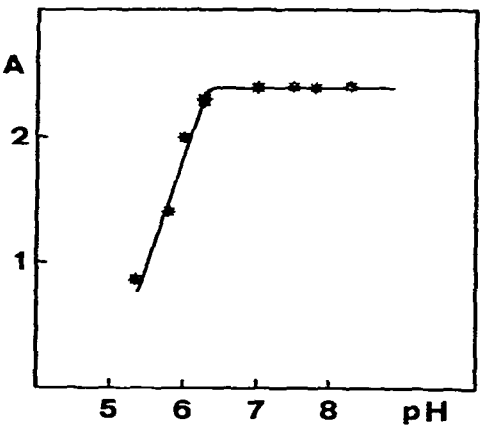

Fig. 6. Eq. 12 (see text) plotted in order to calculate the order of reaction for the dissociation of the PLP-enzyme bond with respect to protons.

can be observed that the reaction is independent of pH at pH > 6.5, while the process has an order of 1.5 with respect to protons at pH < 6.5.

The rise in the rate of reaction when pH is lowered (figs. 1, 4 and 6) could be assigned to an increase in the reactivity of hydroxylamine, but the following facts lead us to believe that this behaviour is due mainly to phosphorylase *b*: (1) Removal of the prosthetic group is accomplished at pH < 6.5 even when a carbonyl group reagent without a p*K* in this region (e.g., L-cysteine) is used [24]. (2) The rate of reconstitution of phosphorylase in the presence of PLP is greatly affected by pH, reaching its peak at pH 6.0 [25]. (3) The behaviour of the fluorescence band of phosphorylase *b* associated with the cofactor as a function of pH is very similar to that observed in fig. 7 [3].

A series of experiments with the Schiff base derivatives obtained by reacting PLP with *n*-hexylamine have been performed in solvents of different polarity, with the purpose of studying the influence of pH upon the PLP-phosphorylase *b* bond.

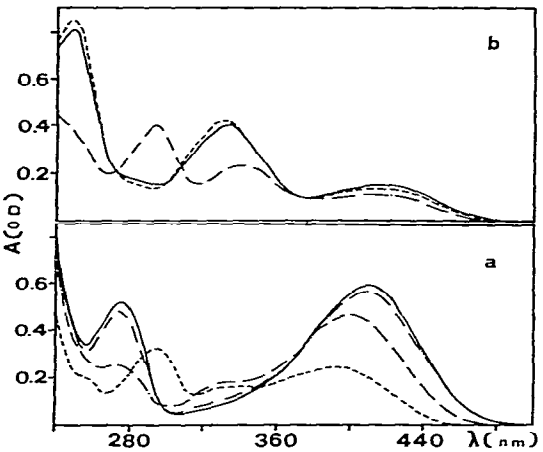

Fig. 7. Ultraviolet-visible spectra of the Schiff base conjugate between 10^{-4} M PLP and 10^{-2} M *n*-hexylamine in ethanol/water mixtures. (a) 15% ethanol (v/v). pH: (———) 7.7, (–·–·–·) 7.4, (– – –) 6.3, (– – – – –) 4.0. (b) 98% ethanol (v/v). pH: (– – – – –) 8.0, (———) 7.0, (– – –) 5.0.

Using solvents with a high dielectric constant (ethanol/water, 15 : 85, v/v), we have obtained a Schiff base conjugate with major peaks at 415 and 275 nm at pH > 7.5 (fig. 7a). When the pH is lowered below this limit, the absorption at 415 nm changes with time, depending on the pH of the solution. When equilibrium is reached, the spectrum shows a maximum at 275 nm less intense than at pH > 7.5 and the major peak at 415 nm is blue-shifted while its intensity decreases in comparison with the value obtained at pH > 7.5. A spectrum with a major 295 nm peak is obtained at pH 4.0, indicating the appearance of free PLP [26].

Identical experiments have been carried out using solvents of low dielectric constant (ethanol/water, 98 : 2, v/v). Under these conditions and at pH > 7.5, the Schiff base derivative of PLP shows bands centred at 250, 333 and 425 nm. At pH < 7, a variation in the absorption at 333 nm with time can be noticed, as occurred in the preceding case. At pH 4.0 the band centred at 295 nm, typical of free PLP, appears in the spectrum. All these considerations point to the existence of an equilibrium between PLP bound to the amine in a Schiff base structure and free PLP, even inside a hydrophobic environment. This equilibrium is pH dependent especially below pH 7.

4. Discussion

Even though removal of PLP seemed to imply the use of deforming reagents such as imidazole citrate of dissociating reagents such as *p*-mercuribenzoate [6,24], it has been proved that the choice of pH and enzyme concentration is the only essential factor.

Our results indicate that the pH must be high enough (pH 7.5) inside the hydrophobic pocket where the coenzyme is located in order to stabilize the Schiff base structure. A drop in the pH of the solution yields distortion of the pocket and subsequent change in the exposure of PLP to the solvent [7]. Under the conditions, our results suggest that the phosphorylase *b*-PLP bond must be partially hydrolyzed, this fact implying an equilibrium between free PLP and PLP bound to the enzyme, in good agreement with the results of Shaltiel et al.

[24], since these authors describe the existence of this equilibrium at pH 6.

Hydroxylamine is a selective reagent for aldehyde groups [27], therefore shifting the equilibrium free PLP-PLP bound to the enzyme towards the first species until complete removal of the cofactor.

The role played by pH in the removal of PLP would be simply to distort the hydrophobic pocket where the cofactor is embedded.

The drop in fluorescence precedes monomerization since dissociation into monomers takes place once the apophosphorylase has been obtained.

It must be emphasized that the same order with respect to the enzyme has been obtained using different techniques (light scattering and fluorescence). The value of this order, -0.3, indicates that we are dealing with a complex process. Hence, removal of PLP occurs in a single stage and the rate of reaction decreases with enzyme concentration.

After the monomerization process, the apoenzyme undergoes self-aggregation leading to species that can be detected using light scattering, though they cannot be studied using fluorescence techniques.

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References

1 A.B. Kent, E.G. Krebs and F.H. Fischer, J. Biol. Chem. 232 (1958) 549.
2 P.J. Kasvinsky and W.L. Meyer, Arch. Biochem. Biophys. 181 (1977) 616.
3 M. Cortijo, I.Z. Steinberg and S. Shaltiel, J. Biol. Chem. 246 (1971) 933.
4 E.H. Fischer, A. Pocker and J.C. Saari, Essays Biochem. 6 (1970) 23.
5 K. Titani, A. Koide, J. Hermann, L.H. Ericson, J. Kumar, R.D. Wade, K.A. Walsh, H. Neurath and F.H. Fischer, Proc. Natl. Acad. Sci. U.S.A. 74 (1977) 4762.
6 C.F. Cori and B. Illingworth, Proc. Natl. Acad. Sci. U.S.A. 43 (1957) 547.
7 S. Shaltiel and M. Cortijo, Biochem. Biophys. Res. Commun. 41 (1970) 594.
8 G.F. Johnson, J.I. Tu, M.L.S. Bartlett and D.J. Graves, J. Biol. Chem. 245 (1970) 5560.
9 J.L. Hedrick, S. Shaltiel and E.H. Fischer, Biochemistry 5 (1966) 2117.
10 S. Shaltiel, J.L. Hedrick, A. Pocker and E.H. Fischer, Biochemistry 8 (1969) 5189.
11 A. Pocker and E.H. Fischer, Biochemistry 8 (1969) 5181.
12 J.M. Vidgoff, A. Pocker, T.L. Hullar and E.H. Fischer, Biochem. Biophys. Res. Commun. 57 (1974) 1166.
13 B. Illingworth, H.S. Jansz, D.H. Brown and C.F. Cori, Proc. Natl. Acad. Sci. U.S.A. 44 (1953) 1180.
14 K. Feldmann and W.E. Hull, Proc. Natl. Acad. Sci. U.S.A. 74 (1977) 856.
15 E.J.M. Helmreich and H.W. Klein, Angew. Chem. Int. Ed. Engl. 19 (1980) 441.
16 E.G. Krebs, D.S. Love, G.E. Gratvold, K.A. Trayser, W.L. Meyer and E.H. Fischer, Biochemistry 3 (1964) 1022.
17 M.H. Buc, A. Ullman, M.F. Goldberg and H. Buc, Biochimie 53 (1971) 283.
18 E.J.M. Helmreich and C.F. Cori, Proc. Natl. Acad. Sci. U.S.A. 51 (1964) 131.
19 R.G. Bates, M. Paabo and R.A. Robinson, J. Phys. Chem. 67 (1963) 1833.
20 R.F. Steiner, L. Greer and R. Bhat, Biochemistry 18 (1979) 1380.
21 F. Muñoz, M.A. Vallés, M. Pocovi, G. Echevarria Gorostidi and F. Garcia Blanco, Biophys. Chem. 18 (1983) 249.
22 F. Muñoz, M.A. Vallés, J. Donoso, G. Echevarria and F. Garcia Blanco, J. Biochem. 94 (1983) 1001
23 S. Shimomura and T. Fukui, J. Biochem. 81 (1977) 1781.
24 S. Shaltiel,J. Hedrick and F.H. Fischer, Biochemistry 5 (1966) 2108.
25 S. Shimomura and T. Fukui, Biochemistry 17 (1978) 5359.
26 E.A. Peterson and H.A. Sober, J. Am. Chem. Soc. 76 (1954) 169.
27 A. Bruylants and E. Feytmants-de Medicis, in: The chemistry of the carbon-nitrogen double bond, ed. S. Patay (Interscience, New York, 1970) p. 468.